Chapter 3

Phenotypic and functional characterization of \textit{AtUGT} knockout mutant of \textit{Arabidopsis thaliana} under abiotic stress

3.1. Introduction

Glycosyltransferases (GTs) are the enzymes responsible for glycosylation of plant compounds. After the analysis of entire \textit{Arabidopsis} genome sequence there were more than 100 putative GTs present in this model plant, which were supposed to be involved in the modification of plant secondary metabolites (Li \textit{et al.} 2001). GTs typically transfer single or multiple activated sugars from nucleotide sugar donors to a wide range of small molecular acceptors of plants. 3β-hydroxy group molecules are the most common acceptors, whilst UDP-glucose is the most common donor. Glycosylation is thought to be one of the most important modification reactions of plant secondary metabolites, which plays a key role in maintaining cell homeostasis and likely to be participating in the regulation of plant growth, development and defense responses to stress environments (Chaturvedi \textit{et al.} 2011). Studies on glycosyltransferases and glycosylation towards low-molecular weight plant compounds have been done mainly on \textit{A. thaliana} and several other plant species. Because of the availability of considerable biochemical and genomic data on plant GTs, the analyses of the biological roles of GTs in plants could be possible by using the techniques of functional genomics, especially the strategies of gene over-expression and gene knockout (or knockdown). The genome of \textit{A. thaliana} contains two sterol β-glucosyltransferase genes in the subfamily UGT80 named as UGT80B1 (\textit{At1g43620}) and UGT80A2 (\textit{At3g07020}), which catalyzes the glycosylation of 3β-hydroxy group of sterols to produce a 3β-D-glycoside. SGs have been found as abundant membrane components both in prokaryotes and eukaryotes since they are important in membrane fluidity and permeability and the phospholipid dependency of UDP-Glc: sterol glucosyltransferase (Bouvier-Nave \textit{et al.} 1984). A difference in the proportion of glycosylated versus acylated sterols were reported in two different solanaceous species under cold acclimation experiment. It has been postulated that SGs may have a role in adaptation to temperature stress (Palta \textit{et al.} 1993). Previously, it was reported by Debolt \textit{et al.} (2010) where mutation in UDP-glucose:sterol glucosyltransferase (UGT80B1) in \textit{Arabidopsis} caused transparent testa
phenotype and suberization defects in seeds. Moreover, gene expression data from available microarray experiments (Genome Cluster Database) suggested that At1g43620 (UGT80B1) transcripts were slightly up-regulated by cold stress. The mutant described here provided an opportunity to test this hypothesis with respect to expression of At1g43620 gene under abiotic stress. Recent results obtained with functional characterization of plant GTs indicated that glycosyltransferases might play an important role in plant growth, development and interaction with the environment.

In the present chapter, we have identified Arabidopsis mutants with T-DNA inserts in At1g43620 gene and determined the effects of the mutations on seed germination, plant growth, development, and environmental stress conditions. Our results indicated that At1g43620 genes are important for normal plant growth and development and influenced the different abiotic stress responses. Differences in the germination percentage and phenotypes of At1g43620 mutant plants suggested that these proteins of wild-type (WT) have distinct as well as common functions, a conclusion reinforced by gene expression profile analysis. At1g43620 mutant plants were remarkably more affected in salt, heat and cold stress as compared to complemented lines of this knockout mutant. This was indicating that Arabidopsis sterol glycosyltransferase (At1g43620) gene may be involved in modulating of sterol/sterolglucoside under adverse environmental conditions.

### 3.2. Materials and methods

#### 3.2.1. Plant materials and growth conditions

All the Arabidopsis thaliana lines used in the present study were of the Columbia background (Col-0). Arabidopsis plants were grown in a controlled culture room set at 22°C under long day (LD) conditions (16-h light and 8-h dark) with white light illumination (120 μmol m⁻² s⁻¹). Seeds were surface sterilized using 30% bleach solution and stratified for 3 d on 0.15% agar at 4°C. For phenotypic analysis and growth assays, seedlings were exposed to light for 1 h and grown in either continuous light or complete darkness at 22°C on plates containing 0.5X Murashige and Skoog’s (1962) mineral salts (Sigma) and 0.8% agar. The T-DNA insertional sterol glycosyltransferase mutants Salk-021175 were obtained from a mutant pool available
from the *Arabidopsis* Biological Resource Center (ABRC, Ohio State University, OH).

### 3.2.2. Homozygous identification of T-DNA insertions in Salk-021175 mutants

Homozygous selection was done by genomic DNA PCR and antibiotic selection for three generations.

**First step**

Before genomic DNA isolation, DNA extraction buffer was prepared for isolation of DNA.

**DNA Extraction Buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris 1M (pH 8.0)</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>EDTA 0.5M</td>
<td>500 µl</td>
</tr>
<tr>
<td>SDS (10%)</td>
<td>500 µl</td>
</tr>
<tr>
<td>NaCl 5M</td>
<td>500 µl</td>
</tr>
<tr>
<td>H2O</td>
<td>6.5 ml</td>
</tr>
</tbody>
</table>

**Second step**

**Genomic DNA isolation**

1- Crushed the leaf in Eppendorf tube for 10 sec with plastic mortar pestles.
2- Added 300 µl DNA extraction buffer in tube.
3- Again crushed up to fully homogenized.
4- Centrifuged the sample at 13,000 rpm for 5 min at room temperature.
5- 250 µl of supernatant was taken and added the same volume (250 µl) of isopropanol.
6- Inverted and mixed for 5 min.
7- Centrifuged the sample at 13,000 rpm for 10 min at 4°C.
8- Precipitated the DNA sample and liquid was discarded.
9- Washed the DNA sample by 70% alcohol and heat dried at 65°C.
10- Dissolved in 30 µl water and mixed thoroughly.
11- Used the sample DNA for PCR analysis with gene specific primers.

The Salk homozygous T-DNA insertion mutation in *At1g43620* (UGT80B1) was identified by PCR as described by Sussman *et al.* (2000). Screening primers for *At1g43620* resulted in 400- and 1000-bp mutant and WT products using T-DNA
primers LBa1 5’-TGG TTC ACG TAG TGG GCC ATC G- 3’, *At1g43620* (Rev) 5’-TAT AAC TTG TAT GAT GCG CTC TAA G -3, and *At1g43620* (Fwd) 5’- TCA AGG TCG GTG CAC CTG CCT T-3’. A 55°C annealing temperature was applied. Primers used for PCR and qRT-PCR analysis in the present studies have been mentioned in appendix II.

3.2.3. **Cloning of *At1g43620* gene in mutant for preparation of complementation line through pIG121 vector with constitutive35S promoter**

For preparation of complementation line (restored), we have cloned *At1g43620* gene coding region (1.84 Kb) into pIG121 vector with 35S promoter (Fig. 3.1A). Cloning was done by different steps given below.

![Figure 3.1A: Schematic diagram showing T-DNA region of binary vector pIG121 used for transformation.](image)

The *At1g43620* gene was inserted at the *XbaI*-SacI site in sense orientation. Promoter DECaMV35S (600 bp), *At1g43620* (1.84 kb) and the restriction sites were assembled and transformed in *A. thaliana*.

3.2.3.1. **Isolation of total RNA**

Total RNA from leaves of *A.thaliana* (Col-0) was extracted using Plant Spectrum RNA Kit (Sigma) following the manufacturer’s protocol.

**Removal of contaminating DNA using DNase I**

Total RNA was treated with RNase-free DNase I according to the manufacturer’s instructions (Sigma).

- Five µg of RNA was given DNase treatment for 15 min at 37°C and reaction was stopped by adding 3 µl of 25 mM EDTA and heating at 70°C for 5 min.
Volume of the reaction was made upto 200 µl with water and extracted with equal volume of chloroform: isoamyl alcohol and centrifuged at 12,000×g for 5 min.

The aqueous phase was transferred to fresh tubes and precipitated with sodium acetate and ethanol by keeping over night at -20ºC.

RNA was pelleted by centrifugation at 12,000×g. The pellet was washed with 70% ethanol, air dried and dissolved in 10 µl of water.

**First strand cDNA synthesis**

The first cDNA strand was synthesized according to manufacturer’s instructions using 4-5 µg of total RNA, 500 ng oligo(dT)_{18} primer and the Superscript™ II RNase H^{-} Reverse Transcriptase (Invitrogen) in a total volume of 20 µl.

### 3.2.3.2. Preparation of cDNA

For cloning of gene usually 4-5 µg RNA was taken for the preparation of cDNA.

Preparation of reaction mixture:-

- RNA (4 µg) + oligo(dT)_{18} primer 1 µl + DEPC treated water = total volume 12 µl
- Incubated at 4ºC for 5 min, chilled on ice, spin down and placed the vial back on ice.

Added the following component in the indicated order.

- 5x reaction buffer = 4.0 µl x 1 = 4 µl
- RNase inhibitor = 1.0 µl x 1 = 1 µl
- 10 mM dNTP = 2.0 µl x 1 = 2 µl

Total volume = 19 µl

Incubated at 37ºC for 5 min

Added the reverse ATH minus reverse transcriptase enzyme (200 u/µl) = 1 µl

Centrifuged for proper mixing

For oligo(dT)_{18} or gene specific primers cDNA synthesis incubated for 60 min at 42ºC

The reaction was stopped by heating at 70ºC for 10 min and chilled on ice.
3.2.3.3. Cloning of \textit{At1g43620} gene into pTZ57R/T cloning vector:

The open reading frame for \textit{At1g43620} was amplified by reverse transcription polymerase chain reaction from mRNA extracted from \textit{Arabidopsis} plants. The genes were first cloned in a pTZ57R/T by designing primers that contain specific restriction sites overhang before ATG at 5’ end of the forward primer and reverse primer. Cloned cassette was transformed into \textit{E.coli} bacteria (DH5α). Positive colonies were selected through blue/white screening. Plasmid was isolated through positive colonies and cloned DNA fragment in pTZ57R/T vector was further confirmed through PCR and sequencing (Fig. 3.1B). Sequencing of the DNA fragments was carried out on automated DNA sequencing system by using stammered M13 primers.

![Figure 3.1B: PCR analysis of positive clones in pTZ57R/T cloning vector. Four positive clones were identified through gene specific primer.](image)

3.2.3.4. Isolation of \textit{At1g43620} gene with \textit{XbaI} and \textit{SacI} site from positive clone by PCR

After confirming the cloned DNA fragment, it was isolated through site specific primers and ligated into the pBI121 binary vector. Ligated product was transformed in \textit{E.coli} (DH5α). Positive transformed bacteria were selected through kanamycin selection marker. Further positive clone was confirmed through PCR and sequencing method (Fig. 3.1C).
3.2.3.5. Transformation of *A. tumefaciens* by binary vector

The expression cassette was constructed using *CaMV35S* enhancer promoter and *At1g43620* gene in pIG121 vector. *A. tumefaciens* strain GV3101 (pMP90) (Koncz and Schell 1986) were transformed with binary vector pIG121 following the modified protocol of “Electroporation of *Agrobacterium*” discussed by Cangelosi *et al.* (1991).

**Preparation of electro-competent cells of *A. tumefaciens***

- The laboratory glycerol stock strain of *A. tumefaciens* was streaked on YEB medium plates containing gentamycin (100 µg/ml) and rifampicin (50 µg/ml) and grown for 48 h at 28°C.
- 5 ml of YEP medium containing same antibiotics was inoculated with a single isolated colony and grown with shaking at 200 rpm for 26-28 h at 28°C.
- 100 µl of this culture was inoculated in 100 ml YEP medium containing same antibiotics and grown till the OD reaches to 0.4-0.6. The culture was centrifuged at 4000 rpm for 5 min at 4°C.
- The bacterial cells were washed with 40 ml of 1 mM HEPES buffer (pH 7.0).
- Cells were pelleted and re-suspended in 40 ml of 1 mM HEPES (pH 7.0) containing 10% glycerol.
- The tube was centrifuged and the cells were re-suspended in 1 ml of 1 mM HEPES (pH 7.0) containing 10% glycerol. The suspended cells were again centrifuged at 4000 rpm for 5 min at 4°C.
The pellet was suspended in 100 µl of above-mentioned buffer and dispensed in aliquots of 50 µl and stored at -80ºC.

### 3.2.3.6. Transformation of *A. tumefaciens*

*A.tumefaciens* transformation was performed by the electroporation using Bio-Rad instrument. Purified pIG121 (2 ng) was added on the top of the 50 µl frozen competent cells and incubated on ice for 2 min. Competent cells/DNA mixture was placed in an ice-cold 0.2 cm electroporation cuvette. The parameters of electroporation were 25 µF, 200 Ω for a 2.5 KV pulse, followed by 5 min delay. One ml of YEP medium was added to the electroporation mixture and transferred to a sterile tube and incubated with shaking at 200 rpm for 2 h at 28ºC. The culture (200 µl) was plated on YEB medium containing gentamycin, rifampicin and kanamycin (100, 50 and 100 mg/l, respectively) and grown at 28ºC for 48 h. Positive clone was determined by plasmid DNA PCR (Fig. 3.1D).

![Image](image.png)

**Figure 3.1D: PCR analysis of positive clone in *A. tumefaciens* cloning vector.**

Single colony was found positive after transformation in *A.tumefaciens*.

### 3.2.3.6. *A. tumefaciens*-mediated transformation of *A. thaliana*

Transformation of *A. thaliana* plant was done by floral dip method (Clough and Bent, 1998).

#### 3.2.3.6.1. Plant growth

*Arabidopsis* plants were grown to flowering stage in a growth chamber (M/s Heraeus Co.) at 22ºC under long day (LD) conditions (16 h light and 8 h dark) with white light illumination (120 µmol m⁻² s⁻¹) provided by fluorescent tubes. Plants were
planted 1-2 per 25 cm² pot. To prevent the soil in larger parts from falling into inoculation medium soil was mounded slightly above the rim of plant container, seeds were planted and soil was then covered with nylon window screen and secured by a rubber band. To obtain more floral buds per plant inflorescences were clipped after most plants had formed primary bolts, relieving apical dominance increasing synchronized multiples secondary bolts.

3.2.3.6.2. Protocol for culture of *A. tumefaciens* and inoculation of plants

1. *A. tumefaciens* strain GV3101 (pMP90) carrying the binary plasmid pIG121 was used in the experiments.
2. 5 ml of LB medium carrying added kanamycin (100 µg/ml) and rifampicin (50 µg/ml) antibiotics was inoculated with a single isolated colony and grown with shaking at 200 rpm for 26-28 h at 28ºC.
3. 100 µl of this culture was inoculated in 100 ml LB medium containing same antibiotics and grown with shaking at 200 rpm for 12 h at 28ºC till stationary phase.
4. Cells were harvested by centrifugation for 20 min at 13,000 rpm at room temperature and then resuspended in infiltration medium to a final OD₆₀₀ of approximately 0.8 were used.
5. Silwet L-77 (0.05%) was used as surfactant because it reduces surface tension more than the most surfactants and at dose with low phytotoxicity and greatly enhances entry of bacteria into relatively inaccessible plant tissues. It was added into infiltration media before dipping.
6. For floral dip, the bacterial suspension was put into the beaker and plants were dipped into this suspension in such a way that all aerial plant tissues were submerged for 10 sec with gentle agitation. Dipped plants were removed from the beaker, then covered with a tall clear plastic dome to maintain humidity.
7. Plants were left in a low light overnight and returned in the growth chamber the next day. Plastic domes were removed approximately 12-24 h after treatment.
8. Plants were grown for further 3-5 weeks until siliques were brown and dry.
9. Seeds were harvested by gentle pulling of grouped inflorescences through fingers over a clean paper. Seeds were stored in microfuge tubes and kept at 4ºC under desiccation.
3.2.3.6.3. Selection of transformants using antibiotics

Seeds were sterilized by liquid sterilization method. Seeds were first treated with 95% ethanol for 30-60 sec, then with 50% bleach (2% sodium hypochlorite) containing 0.05% Tween-20 for 5 min followed by rinses with sterilized water for 5 times. To select the transformed plants, sterilized seeds were placed on hygromycin (20 µg/ml) selection plates. Placed the dishes at 3-4°C (refrigerator temperature) for at least 2 to 4 days to break dormancy and then grown for 7-10 days in controlled environment at 20°C under long day (LD) conditions (16 h light and 8 h dark) with white light illumination (120 µmol/m² s⁻²) provided by fluorescent tubes. Selection plates contain ½ MS medium, 0.8% agar with 20 µg/ml hygromycin. Positive lines on selected plates were further confirmed by PCR analysis and homozygous lines were determined through T3 generation.

3.2.3.6.4. Genomic DNA isolation for confirmation of positive lines

For confirmation of positive transgenic lines, we have isolated genomic DNA as described previously in cloning section. Positive lines were confirmed through antibiotic selection as well as gene specific primers or antibiotic specific primers (Fig. 3.1E).

![Figure 3.1E: Selection of positive transgenic lines through gene specific PCR.](image)

Figure 3.1E: Selection of positive transgenic lines through gene specific PCR. Figure shows confirmation of two positive transgenic lines through PCR after hygromycin selection.
3.2.4. Transcript level analysis

The modulation of *AtSGT* transcript levels were measured by real time qRT–PCR. Total RNA samples were extracted from appropriate plant materials using the RNeasy Plant Total RNA Isolation Kit (Qiagen, USA). Prior to qRT–PCR, total RNA samples were extensively pre-treated with RNase-free DNase I (Sigma) to eliminate any contaminating genomic DNA. The first-strand cDNA was synthesized from 2 µg of total RNA in a 20 µl reaction volume using Superscript II reverse transcriptase (Invitrogen). The *AtSGT* gene expression was normalized to *Actin* (At3g18780) as an internal (housekeeping gene) control along with –RT and non-template controls. All the qRT–PCR runs were repeated at least three times and a representative result was displayed for individual assays.

3.2.5. Histochemical Analysis

3.2.5.1. Tetrazolium Salt Uptake

Ability of seeds to uptake salt was tested by placing whole seeds in an aqueous solution of 1% (w/v) tetrazolium red (2, 3, 5-triphenyltetrazolium) at 30°C for 4 to 24 h. Seeds were removed from the solution and imaged by light microscopy.

3.2.5.1. DMACA

Seeds were stained with DMACA reagent (2% [w/v] DMACA in 3 M HCl/ 50% [w/v] methanol) for one week, and then washed three times with 70% (v/v) ethanol. The stained pools were then examined using light microscopy.

3.2.6. Effect of salt stress

Seeds of wild type (WT) *Arabidopsis* plants, the knockout mutants and *p35S:At1g43620* complemented lines of *A. thaliana* were germinated on ½ MS media with 2% sucrose and 0.85% agar in a controlled culture room set at 22°C under long day (LD) conditions (16-h light and 8-h dark) with white light illumination (120 µmol m⁻² s⁻¹) provided by fluorescent tubes. For providing salt stress, NaCl was added to the medium at 50, 100, 150 and 200 mM concentrations, while water (0 mM NaCl) was taken as the control. The observed parameters were: 1) germination percentage, 2) root length, 3) fresh weight, 4) dry weight, and 5) chlorophyll content.
level of \textit{At1g43620} gene was quantified by real time-PCR under normal conditions in 14-days-old WT plants grown on $\frac{1}{2}$ MS at 0 (water), 50, and 100 mM NaCl after 24 h.

### 3.2.6.1. Relative electrolytic conductivity

After salt stress the relative electrolyte conductivity (REC) was measured for all phenotypes to assess stress adaptation as by Mishra \textit{et al.} (2013). REC was measured for each sample with a conductivity meter before and after autoclaving (121°C for 20 min). Fully developed leaves of salt treated or non-treated plants were excised from the base of the petiole and placed in 13x100 mm glass tubes containing 100 μl of deionized water. The tube containing the solution was shaken and left overnight at 24°C. The conductivity of the solution was measured in each tube and the tubes were capped (to minimize evaporation), autoclaved and cooled. After autoclaving, the conductivity of the solution was again measured in each tube. The percentage of electrolyte leakage was calculated as the ratio of the conductivity before autoclaving to that after autoclaving. It is assumed that the conductivity after autoclaving represents complete (100%) electrolyte leakage.

### 3.2.6.2. Measurements of oxidative stress, SOD enzyme activity

Total amount of SOD enzyme was estimated as a function of NBT reduction using spectrophotometer. Leaf samples (250 mg) from the seedlings were homogenized in a precooled mortar in homogenizing buffer.

**Extraction buffer**

\[
\begin{aligned}
\text{EDTA} &\quad - 0.1 \text{ mM} \\
\text{Triton-X 100} &\quad - 0.5\% (\text{v/v}) \\
\text{PVP} &\quad - 1\% (\text{w/v})
\end{aligned}
\]

**Phosphate buffer** – 100 mM (pH 7.8)

The homogenate was transferred to 1.5 ml Eppendorf tubes and centrifuged at 13,000 rpm for 20 min at 4°C. The supernatant was collected and total SOD levels were estimated according to Beyer and Fridovich (1987). Protein content was estimated according to the dye binding method of Bradford (1976). The total SOD activity was measured by adding 20 μl supernatant to a reaction mixture containing 4.4% (w/v) Riboflavin, 57 μm NBT, 10 mM L-Methionine and 0.025% (v/v) Triton-X 100 in 100
mM phosphate buffer. One unit of enzyme activity was defined as the amount of enzyme required for 50% inhibition of NBT reduction in 2 min at 25°C.

3.2.7. Effect of heat stress

3.2.7.1. Expression analysis of *At1g43620* gene under heat stress

During heat stress, expression of *At1g43620* gene was determined in WT plants by qRT-PCR. WT plants were grown for 14 days under 16 h /8 h light/dark and the treatment was given at 37°C for 1h and 3h. Zero h represented as control condition. Leaves after the treatment, were harvested immediately and stored at -80°C.

3.3.7.2. Heat sensitivity at 14-d-old seedling stage

Thermotolerant/sensitive phenotype was observed by Larkindale *et al.* (2005) method. For phenotypic observation under heat stress both WT and overexpression lines of *A. thaliana* seedlings were grown on agar plates in light for 14 d and heated to 42°C for 180 min (basal thermotolerance). Percentage of survival of plants in relation to WT control plants on the same plate was determined 7 d after heat stress.

3.2.7.2. Lipid peroxidation assay

Analysis of lipid peroxidation assay by malondialdehyde (MDA) content which is a measure of heat stress was performed as described by the protocol by Larkindale *et al.* (2005). Plants were grown on agar plates. Seedlings were heated to 42°C for 60 min and left to recover under normal light conditions for 2 days. Leaf tissue (100 mg) was homogenized by adding 0.5 ml 0.1% (w/v) trichloroacetic acid (TCA). Homogenate was centrifuged for 10 min (15,000 g at 4°C) and the supernatant was collected. The 0.5 ml of supernatant was mixed with 1.5 ml of 0.5% thiobarbituric acid (TBA), diluted in 20% TCA, and was incubated in water bath at 95°C for 25 min. Finally, it was incubated on ice to complete the reaction. The absorbance was measured at 532 and 600 nm. The values of OD<sub>600</sub> were subtracted from the MDA-TBA complex values at 532 nm and MDA concentration was calculated using the Lambert-Beer law with an extinction coefficient ε<sup>M</sup>=155 mM<sup>-1</sup> cm<sup>-1</sup>.

3.2.8. Cold stress and determination of freezing tolerance by survival rate

For cold stress, transcript level of *At1g43620* gene was analyzed by real time-PCR. All phenotypes were grown for 14 d in controlled chamber at 22°C under constant
light (120 μmol m⁻² s⁻¹) and cold treatment was given at 4°C for 24 h. Monitoring of plant survival by whole plant freezing test based on the protocol of Xin and Browse (1998) was performed separately with the seedlings grown on petriplates.

3.2.9. **Chlorophyll estimation of all phenotypes**

Estimation of chlorophyll in all normal grown plants and treated plants was done by Ritchie (2008) protocol. In the present study, 80% acetone was used for determination of chlorophyll and absorption was recorded at 645 and 663 Å.

3.2.10. **Chlorophyll fluorescence measurements for finding the effect of transgene**

An Imaging-PAM, M-Series Chlorophyll Fluorometer (Walz, Effeltrich, Germany), was used to study the chlorophyll fluorescence parameters. Calculations of various chlorophyll fluorescence parameters were done according to Maxwell and Johnson (2000). The maximum photochemical efficiency of photosystem II (PSII), (Fv/Fm: where Fm is maximum fluorescence of the dark-adapted leaf under a light saturating flash and Fv is maximum variable fluorescence, Fm – F0) was measured on leaves after 20 min of dark adaptation. The effective quantum yield of PSII (Y) was calculated as (Fm-Fs)/Fm. NPQ is a non photochemical quenching measurement that indicates a change in efficiency of excess excitation energy dissipation by heat. NPQ collectively indicates heat dissipation triggered by low thylakoid lumen pH, state transitions of PSII centers, and photo inhibition. Y (NPQ) is a measure of the fraction of photons absorbed by PSII antennae. Increased NPQY is an indication of protective strategies at PSII. Y (NO) is the fraction of photons dissipated by dissociation of light-harvesting complex II and indicates irreversible PSII damage.
3.3. Results

3.3.1. *In silico* analysis of *At1g43620* gene

*At1g43620* gene was presented on chromosome 1 ([www.cazy.org](http://www.cazy.org)) and catalyzes the glycosylation of 3β-OH sterols in *Arabidopsis* (Fig. 3.2).

![Figure 3.2: Chromosome localization study of *AtUGT* gene. *AT1g43620* (*UGT80B1*) is localized on chromosome -1. This map was taken from Cazy database.](image)

To explore the phenotypic characterization of *At1g43620* mutant, we first investigated expression of *At1g43620* gene at different plant developmental stages and under different abiotic stress conditions for knowing the expression pattern using a DNA microarray database. Gene expression data from available microarray experiments NASCArrays ([www.affymetrix.arabidopsis.info](http://www.affymetrix.arabidopsis.info)) suggested that *At1g43620* transcripts were slightly up-regulated by cold stress, heat stress and salt stress. GENEVESTIGATOR software showed the involvement of sterol glycosyltransferase gene in plant growth and development which was strongly induced during cold stress (Fig. 3.3A, 3.3B). These all *in silico* data suggested *AT1g43620* gene is important for plant growth as well as environmental stress, so we have further characterized this gene through reverse genetic screening.
Figure 3.3 A: Genevestigator analysis of *At1g43620* gene in different plant parts. Heat map showed *At1g43620* gene was highly expressed in inflorescence and shoot.

Figure 3.3 B: Genevestigator analysis of *At1g43620* gene in different abiotic stress. Heat map showed *At1g43620* gene poorly expressed in salt stress and heat stress as compared with the control. Enhanced expression of *At1g43620* gene under cold stress.

### 3.3.2. Molecular characterization and phenotypic analysis of *At1g43620* mutants

To study the modulation of *AtUGT* gene under abiotic stress conditions, we obtained T-DNA insertion lines from the *Arabidopsis* Biological Resource Center and identified homozygous plants. The initial allele that we obtained, were designated as Salk-021175, contained T-DNA insertion at the fifth exon, downstream of the translational initiation codon (Fig. 3.4A).
T-DNA insertion at fifth exon position in *At1g43620* gene was designated as Salk-021175 mutant. Gray boxes represent the position of exon and gaps represent the position of intron.

Homozygous lines carrying T-DNA mutations in Salk-021175 were isolated by PCR analysis with kanamycin selection (Fig. 3.4B). RT-PCR analysis showed that absence of gene expression in homozygous line due to T-DNA insertion (Fig. 3.4C).

**Figure 3.4B-C:** Molecular characterization of Salk-021175. B. PCR analysis of genomic DNA of knockout mutant Salk-021175 and WT. Lane 5 represents homozygous line carrying T-DNA insertion in both alleles, while Lane 7 represents WT. C. RT–PCR analysis, total cellular RNA from WT and homozygous knockout mutant.

Homozygous *AtUGT* (*At1g43620*) mutant plants showed the effects of absence of this gene at several stages of plant development. The Salk-021175 phenotype was characterized by normal height, light green color and smaller rosette area compared to WT (Figs. 3.5B, 3.5E). The cotyledons of mutant seedlings were smaller, dark green, and epinastic (i.e., curled down) (Fig. 3.6H). The hypocotyls of
the homozygous mutant seedlings, with an average length of 3 mm after 7 days, were shorter than WT hypocotyls, which were 6 mm in length (Fig. 3.6E). However, 35S:At1g43620 hypocotyls were 7 mm of average length after 7 days (Fig. 3.6I). The root systems in mutant plants were shorter than that in WT plants, with an ectopic root hair (Fig. 3.7) and the cells in the elongation zone were markedly smaller as visible under microscope. The floral organs, particularly petals and stamens, were also affected in mutant plants. The flowers opened maturely, with small petals that curled outward and stamens were shorter than the carpels in the knockout mutants as compared to WT as well as 35S:At1g43620 (Fig. 3.8A-C). Thus the fertilization of homozygous mutant plant resulted in small siliques having less number of seeds (Fig. 3.8D-F). The most notable phenotypic feature was, Salk-021175 displayed a transparent testa phenotype and a reduction in seed size (Fig. 3.9A). Due to reduction in seed size seed weight also decreased in salk-021175 mutant (Fig. 3.9B). A phenotypic feature of rescued p35S::At1g43620 line was almost similar to WT but growth of plants was retarded as compared to WT (Figs. 3.5C&F, 3.6I-L, 3.7, 3.8C&F, 3.9B)

**Figure 3.5:** Morphological differences in WT, homozygous mutant and complementation line of *A. thaliana*. A&D. 3 weeks and 5 weeks old potted WT plant. B&E. 3 weeks and 5 weeks old potted *AtUGT* mutant plant with small rosette area and small leaf size. C&F. 3 weeks and 5 weeks old potted 35S:At1g43620 complemented plant with similar morphology like WT plants.
Figure 3.6: Seed germination (From L to R) of *Arabidopsis* after 2, 3, 5 and 7 days. **A-D.** Germination of WT seeds in ½ MS medium. **E-H.** Germination of Salk-021175 seeds in ½ MS medium. **I-L.** Germination of 35S:At1g43620 transgenic *Arabidopsis* seeds in ½ MS medium.

Figure 3.7: Phenotypic differences of roots of WT, homozygous mutant and complementation line of *A. thaliana* (From L to R) WT (Col-0), Salk-021175 and 35S:At1g43620. Salk-021175 mutant was showing ectopic root hair in root.
Figure 3.8: Phenotypic differences of flowers and siliques in WT, homozygous mutants and complementation line of *A. thaliana* (From L to R) WT (Col-0), Salk-021175 and 35S:*At1g43620*. A-C. Flowers showing petals and shorter stamens than the carpels in mutant line. D-F. Siliques, smaller size in mutants with lesser number of seeds.

Figure 3.9: Phenotypic differences of seed and seed weight in WT, homozygous mutant and complementation line of *A. thaliana*. A. Light color seed coat present in Salk-021175 mutant, complemented line rescued. B. Average seed weight less in mutant as compared to WT and complemented line.
3.3.3. Histochemical and microscopic analysis of seeds

A visual inspection showed that seeds of *AtUGT* mutant were reduced in size and seed coat was light in color as compared to WT. To know the possible reason for increased permeability of mutant seeds, we have done a series of histochemical and microscopic analysis of seeds similar to Debolt *et al.* (2010). When seeds were treated in a solution of tetrazolium salt, mutant seeds were observed to be highly sensitive to salt uptake but unable to limit uptake and entire seeds became stained with dark red color, whereas WT seeds absorbed small amount of salt (Fig. 3.10A). Phenotype of *p35S:At1g43620* seeds behaved like WT means absorbed small amount of salt (Fig. 3.10A). Pigmentation of the seed coat was determined by the deposition of flavanoids using 4-dimethylamino cinnamaldehyde (DMACA) reagent. Consistent with the transparent testa phenotype, the most drastic reduction in DMACA staining was in mutant. The DMACA staining phenotype of *p35S:At1g43620* seeds were also rescued (Fig. 3.10B).

![Figure 3.10: Histochemical analyses of seeds in WT, homozygous mutant and complementation line of *A. thaliana.* A. Tetrazolium salt uptake, Salk-021175 was displayed with a transparent testa phenotype. B. DMACA staining showing altered deposition of flavonoid on seed coat.](image)
3.3.4. Spatial and temporal regulation of \textit{At1g43620} genes

To obtain more insights into the functional role of \textit{AtUGT} proteins, spatial and temporal expression patterns of the \textit{AtUGT} genes were systematically examined. Interestingly, the \textit{AtUGT} genes exhibited distinct tissue-specific expression patterns. \textit{AtUGT} transcripts could be detected by qRT–PCR under normal growth conditions, \textit{At1g43620} were expressed at relatively high levels in the siliques, flower and shoots but their transcript levels were very low in the roots (Fig. 3.11A). This expression pattern was similar like as GENEVESTIGATOR software analysis. It is currently unclear whether these distinct patterns of spatial expression of \textit{AtUGT} genes are related to modulation of sterol glycosides under different abiotic stress conditions. Individual \textit{AtUGT} genes also exhibited dynamic expression patterns throughout the growth stages. Transcript levels of the \textit{AtUGT} genes were relatively very low in young seedlings (up to 7 days after germination; DAG) (Fig. 3.11B). However, the transcript levels of \textit{At1g43620} were significantly elevated after that time period. \textit{Atlg43620} were gradually increased as the plants grew, reaching a plateau at around DAG 14 (Fig. 3.11B). Together, these observations suggest that the spatial and temporal expression patterns of the \textit{AtUGT} genes may be intimately linked to their functional roles in plant development and modulation under different stress conditions.

![Figure 3.11: Spatial and temporal transcript expression of \textit{AtUGT} genes by qRT–PCR. A. Quantitative representation of relative expression of \textit{At1g43620} gene under normal growth conditions in root, stem, leaf, flower and silique. B. Quantitative representation of relative expression of \textit{At1g43620} gene in development stage.](image)


3.3.5. Expression analysis of At1g43620 transcripts under abiotic stress

NASC array data showed that At1g43620 transcript was induced slightly under salt treatments and heat stress, whereas under cold stress this gene was highly expressed. To assess whether At1g43620 were actually expressed under salt, heat and cold stress, qRT–PCR analysis was performed by using At1g43620 specific primers after salt, heat and cold treatments on 14-days-old seedlings. As shown in Fig. 3.12A, At1g43620 was expressed relatively more 3.5 fold at 50 mM NaCl (for 24 h). When the salt concentration increased upto 100 mM NaCl transcripts gradually decreased in 24 h. These results indicated that At1g43620 gene was induced by little amount of salt stress conditions whereas unable to show the expression at higher concentration. In an another experiment when the plants were treated at 37°C heat stress for 1h and 3h, expression of At1g43620 gene was slightly increased upto 1h but when time period was increased expression was declined (Fig. 3.12B).

![Figure 3.12: Expression analyses of At1g43620 transcripts under salt concentration and heat stress: A. Quantitative representation of the relative expression of At1g43620 gene under 50 mM and 100 mM salt stress, 0 mM NaCl or water acted as control. B. Quantitative representation of the relative expression of At1g43620 gene under heat stress for 1h and 3h. Values are mean ± SE, n = 3, (*) for P≤0.05, (**) for P≤0.01, (***) for P≤0.001 or 0.005, significantly different from the control (t-test)](image)

For analysis under cold stress conditions, Arabidopsis plants were grown for 14 days in controlled chamber at 22°C under constant light (100 μmol m⁻² s⁻¹) and cold treatment was given at 4°C for 24 h. Arabidopsis leaves were collected at 0, 12
and 24 h, where 0 h were treated as control. The \textit{At1g43620} expression level was increased 18 fold upto 24 h. (Fig. 3.12C). This result suggested that \textit{At1g43620} was induced by cold stress condition and the expression patterns of this gene matches with that of available microarray data.

![Figure 3.12C: Expression analyses of \textit{At1g43620} transcript under cold stress:](image)

Quantitative representation of the relative expression of \textit{At1g43620} gene during a low-temperature (4°C) time course. Values are mean ± SE, n = 3, (*) for P≤0.05, and (***)) for P≤0.001 or 0.005, significantly different from the control (t-test)

### 3.3.6. Effect of salt stress on \textit{At1g43620} mutant, and \textit{p35S:At1g43620} plants

We have evaluated the phenotypic and physiological changes that occurred in mutants and complemented plants of \textit{A. thaliana} under salt stress. \textit{AtUGT} mutant lines and \textit{p35S:At1g43620} complemented lines germinated well at 50 and 100 mM NaCl. At higher concentration of NaCl (150 and 200 mM), there was no germination of \textit{AtUGT} mutant in contrast to WT seeds and \textit{p35S:At1g43620} complemented lines which showed remarkably high germination (Fig. 3.13A). Germination percentage was calculated as number of seeds germinated per sixty seeds. Also, at 100 mM of NaCl, the germination of mutants was delayed. The cotyledons of Salk-021175 seedlings were smaller, yellowish green (Fig. 3.14 E-H) in colour. The hypocotyls of the mutant seedlings were shorter than the WT hypocotyls. Root length of Salk-021175 knockout mutant was significantly less as compared to WT plants (Figs. 3.13B, 3.15). Root length of \textit{p35S:At1g43620} complemented lines was significantly more than Salk-021175 mutant and almost similar like WT (Figs. 3.13B, 3.15). Fresh weight/dry weight (both fresh weight and dry weight were measured to ascertain the biomass) and number of true leaves were more in \textit{p35S:At1g43620} complemented lines - than
knockout mutants (Fig. 3.13C-D). These results suggested that \textit{AtUGT} mutants germination, root growth, fresh weight and dry weight were more affected under salt stress conditions as compared to normal medium, its complemented phenotype rescued similar phenotypic pattern like WT (Figs. 3.13A-D, 3.15). Therefore, it was hypothesized that \textit{AtUGT} mutant was more affected under salt stress with respect to seed germination and plant development both, whereas, after complementation of \textit{At1g43620} gene in mutant plant, transgenic lines showed better germination and growth. It means \textit{At1g43620} gene expression was not directly involved to the salt stress condition but helps the plants to improve in germination and development under low salinity conditions.

Figure 3.13: Effect of salt stress on WT, Salk-021175 mutant and \textit{p35S:At1g43620} plants. \textbf{A}. Germination percentage of seeds upto 7 days in 0, 50, 100, 150 and 200 mM salt concentrations, 0 mM represent ½ Ms without NaCl. \textbf{B}. Root length of 14-day-old seedlings in different salt concentrations. \textbf{C}. Fresh weight of whole plants measured after 14-days of salt stress. Values are mean ± SE, n= 10. \textbf{D}. 

Dry weight of whole plants measured after 14-days of salt stress. Values are mean ± SE, n=10.

**Figure 3.14:** Seed germination (From L to R) of *Arabidopsis* in presence of 100 mM NaCl after 2, 3, 5 and 7 days. **A-D.** Germination of WT seeds in ½ MS medium with 100 mM NaCl. **E-H.** Germination of Salk-021175 seeds in ½ MS medium with 100 mM NaCl. **I-L.** Germination of 35S:*At1g43620* transgenic *Arabidopsis* seeds in ½ MS medium with 100 mM NaCl.
Figure 3.15: Phenotypic differences (From L to R) in WT (Col-0), homozygous mutant (Salk-021175) and 35S:At1g43620 complementation line of A. thaliana under salt stress. A. Comparison of shoot length, root growth and leaf growth on MS medium. B. With 100 mM NaCl. C. With 150 mM NaCl. Photographs were taken after 14 days of germination.

3.3.7. Measurement of oxidative stress - Comparison of SOD activity and relative electrolytic conductivity (REC) in mutant, and p35S:At1g43620 plants

The p35S:At1g43620 complemented lines expressing the At1g43620 protein remained green but the knockout mutants were wilted when exposed to higher NaCl in comparison to WT plants. The visual observations were confirmed by measuring SOD activity under normal conditions and after three weeks of growth on 100 mM NaCl. Under normal conditions, the level of SOD was almost similar in all the phenotypes as compared to WT plants. Under salt stress, the level of SOD increased in all the phenotypes but in AtUGT mutant, SOD activities were not increased remarkably (Fig. 3.16A). The percentage SOD activity was less in AtUGT mutant as compared to WT
under 50 mM and 100 mM NaCl, respectively, but in case of \textit{p35S:At1g43620}, SOD activity was increased up to 24% more than in WT in 50 mM NaCl, whereas, at higher concentration SOD activity was not much increased as compared to WT. Additionally, comparison of REC between knockout mutant, complemented lines and WT plants showed that at 100 mM NaCl, Salk-021175 plants had a higher REC (101%) than the other phenotypes (Fig. 3.16B). Complemented \textit{p35S:At1g43620} lines and WT plants showed significantly lower REC as compared to \textit{AtUGT} mutants after salt stress. Higher SOD activity at lower concentration of salt and lower relative electrolytic conductivity (REC) reflected that \textit{p35S:At1g43620} lines were adapted to salt stress at low concentration but at higher concentration or long exposure of the salt this gene was not involved for tolerance mechanism.

![Figure 3.16](image)

**Figure 3.16:** SOD activity and relative electrolyte conductivity under salt stress. 
A. SOD activity analysis in WT (Col-0), Salk-021175, and \textit{p35S:At1g43620} under 0, 50 and 100 mM salt stress. Values are expressed as mean (n = 3); errors bars show the SD for each experiment. B. REC analysis under 0, 50 and 100 mM salt stress. Values are expressed as mean (n=3); error bars show SD.

### 3.3.8. Effect of heat stress on \textit{At1g43620} mutant, and \textit{p35S:At1g43620} complemented plants

Phenotypic analysis of \textit{AtUGT} mutant and \textit{p35S:At1g43620} of \textit{A. thaliana} showed heat sensitivity at 14-d-old seedling stage in petriplates (Fig. 3.17). We have found 10-13% survival of \textit{At1g43620} mutant whereas survival percentage of \textit{p35S:At1g43620} transgenic plants enhanced up to 42% similar like WT. Leaves of the
knockout mutant were more bleached as compared to complemented lines after heat stress.

![Figure 3.17: Survival analysis of (From L to R) WT, homozygous mutants and complementation line of *A. thaliana* under heat stress. Seedlings were grown on agar plates in light for 14 d and heated at 42°C for 3h, (basal thermotolerance). Percentage of survival of plants in relation to WT control plants on the same plate was determined 5 d after heat stress.]

**3.3.9. Lipid peroxidation assay**

As a test of heat sensitivity and measure of oxidative damage, heat stressed WT and transgenic lines of *A. thaliana* were assayed for accumulation of MDA. MDA content was 77.96% more accumulated in mutant line as compared with normal MDA level, whereas 65% MDA level was elevated in complemented line and 63.61% in WT. This result suggested that due to heat stress maximum peroxide activity occurs in mutant as compared with complemented and WT lines (Fig. 3.18).

![Figure 3.18: Lipid peroxidation assay of WT, homozygous mutants and complementation line of *A. thaliana* during heat stress. Plants were heat treated as]
described in Figure (3.17), and after 2 days of recovery, seedlings were harvested and stored in liquid nitrogen until the assay was performed. The MDA level was determined. Values are means ± SD (n = 3) (*) for $P \leq 0.05$, (**) for $P \leq 0.001$ or 0.005, significantly different from the control (t-test).

3.3.10. Effect of cold stress on Salk-021175 and p35S:At1g43620 plants

Phenotypic analysis for basal cold tolerance (NA, non acclimated), was performed in growing plates of 14-days-old seedlings of WT, AtUGT mutants and complemented p35S:At1g43620 lines of A. thaliana which were transferred directly to a freezing chamber at -1°C. For cold-acclimated (CA), the plates were first transferred to a cold room set at 4 ± 2°C, under constant light for 7 days. Under NA condition, survival of AtUGT mutants (19.98%) were significantly less than WT plants (35.5%) and complemented p35S:At1g43620 lines (36.67%) (Fig. 3.19). We explored the ability of the plants to acclimatize to cold conditions by measuring ion leakage and survival rates after one week of acclimation at 4°C. Whereas, in case of CA condition, survival percentage of complemented p35S: At1g43620 line upto 78% which was more than AtUGT mutants and WT plants (Figs. 3.19, 3.20A). AtUGT mutant showed 50% ion leakage (LT50) early at -6°C whereas WT and complementation line showed upto -8°C (Fig. 3.20B). To explain the reason for enhanced tolerance against cold stress in complemented line, we assessed a change of typical marker gene, cor15a and Rd29a, which is well known to be involved in cold acclimation process. Both cold responsive gene expressions was higher in complemented line than that of mutant line (Figs. 3.20A, B). These observations suggested that SGT gene of Arabidopsis play a role in the cold acclimation process.
Figure 3.19: Survival analysis of (From L to R) WT, homozygous mutants and complementation line of *A. thaliana* under cold stress. Cold stress was imposed on after 14 days of growth. Plates can be transferred directly to a freezing chamber set at \(-1 \pm 0.1^\circ\text{C}\) in the dark to check the basal cold tolerance (NA). For cold acclimation (CA), the plates were first transferred to a cold room set at \(4 \pm 2^\circ\text{C}\), under constant light for 7 days. Phenotypes of plants after 5 days score the survival of seedlings visually after cold acclimation (CA).

Figure 3.20: A. Survival percentage and constitutive freezing tolerance (LT_{50}) of WT, homozygous mutants and complementation line of *A. thaliana* in NA and CA seedlings. Percentage of survival of plants in relation to WT control plants on the same plate was determined 5 d after cold stress. B. After CA treatment temperature
was decreased at a rate of 1°C per hour until it reached -10°C and calculated LT50. LT50 of Salk-021175 mutant was -6°C. Values are means ± SD (n = 3) (*) for P≤0.05, (**) for P ≤ 0.01, (***) for P≤ 0.001 or 0.005, significantly different from the control (t-test).

Figure 3.21: Expression analyses of Cor15a and Rd29a transcripts under cold stress: A. Quantitative representation of the relative expression of Cor15a gene during a low-temperature (4°C) time course. B. Quantitative representation of the relative expression of Rd29a gene during a low-temperature. Values are mean ± SE, n = 3, (*) for P≤0.05, and (*** for P≤0.001 or 0.005, significantly different from the control (t-test)

3.3.11. Differential effects of salt and cold treatments on chlorophyll and fluorescence response (Fv/Fm) in At1g43620 mutant, and p35S:At1g43620 plants

Chlorophyll measurement showed more dramatic chlorophyll loss in the mutant leaf disks than the WT and p35S:At1g43620 complemented lines under salt stress. There was no significant difference in chlorophyll contents between knockout mutant, and p35S:At1g43620 complemented lines without treatment while chlorophyll content was slightly more in WT. However, in the presence of 100 mM NaCl, there was 95% chlorophyll loss in AtUGT mutant leaf disks, but only 46% in complemented phenotypes – the discrepancy was greater with increased NaCl concentrations (Fig. 3.21A). Similarly greater chlorophyll loss occurred during heat stress in AtUGT mutant because after survival maximum leaves were bleached (Fig. 3.21A). However, in the presence of cold stress, there was 22.78% chlorophyll loss in AtUGT mutant leaf
disks, but only 14.67% in complemented phenotypes (Fig. 3.21A). Additionally, further chlorophyll fluorescence imaging has been shown under the selected fluorescence parameters. Variability of the fluorescence response (Fv/Fm) has been shown under salt, heat and cold stress. Under normal conditions, all the genotypes showed approximately similar fluorescence response while under salt stress, fluorescence response gradually decreased in knockout mutant as compared to WT and \textit{p35S:At1g43620} complemented lines. Fluorescence response also decreases in \textit{AtUGT} mutant during heat stress due to maximum chlorophyll loss in mutants as compared to complemented lines and WT (Fig. 3.21B). In contrast, under cold stress fluorescence response also decreased due to chilling injury (Fig. 3.21B). Other fluorescence parameters were also observed like yield (Yll), Y (NPQ), Y (NO) and ETR1 in salt, heat and cold stress. These have followed same response like as Fv/Fm. Y (NO) was increased greatly under salt stress (Fig. 3.21C-F). These results suggested that expression of \textit{p35S:At1g43620} in \textit{Arabidopsis} enhanced its photosynthetic abilities and fluorescence response under different abiotic stress but it was remarkably enhanced during cold stress in complementation line.

![Image A](imageA.png)

![Image B](imageB.png)

![Image C](imageC.png)

![Image D](imageD.png)
Figure 3.22: Chlorophyll contents and study of fluorescence parameters in WT (Col-0), Salk-021175 and 35S:At1g43620 under normal growth condition, salt stress (100 mM), heat stress (42°C) and cold treatment (4°C). A. Total chlorophyll, estimated from equal amount of leaf samples (triplicate in each sample), with and without treatment were represented as mg g\(^{-1}\) FW. B. Fv/Fm measurements. C. Fraction of photons absorbed by PSII antennae Y (NPQ). D. Y (NO) is the fraction of photons dissipated by dissociation of light-harvesting complex II and indicates irreversible PSII damage. E. The effective quantum yield of PSII (Y). F. Electron transport rate through PSII (ETR1). Values are expressed as mean (n = 3); error bars show SD for each experiment.

3.4. Discussion

The physiological importance of sterols in higher plants is their involvement in structural components of membranes and controlling the permeability of membranes. Sterols in higher plants occur in at least three different forms: as free sterols, steryl esters, and steryl glycosides, and the steryl glycosides may or may not be acylated (Grunwald 1971). Biosynthesis of sterol glycoside was done by UDP-sterol glycosyltransferase enzyme. Most of these studies show a correlation between alterations in physiological or morphological parameters and changes in the amount/proportion of steryl glycosides in the respective organism. But no causal connection could be drawn between SGs and any biological phenomenon on a molecular level. In the present work, T-DNA knockout mutant in At1g43620 gene (Salk-021175) and their complemented lines 35S:At1g43620 were phenotypically characterized under salt, heat and cold stress conditions. This AtUGT gene was
examined in this study exhibiting both dynamic and static expression patterns in different plant tissues during all the developmental stages. *AtUGT* gene was expressed with high transcript levels in the siliques and flowers, and moderate transcript levels in the root and leaves. These observations further support that the AtUGT proteins may play certain roles in plant development. Due to T-DNA insertion knockout mutants having sterol glycosides (SG) and acyl sterolglycosides (ASG) were significantly reduced (Debolt *et al.* 2010). Phenotypic observations revealed that T-DNA insertion in *At1g43620* gene caused transparent testa in *AtUGT* mutant. This observation was further confirmed by presence of ectopic root hair (Galway *et al.* 1994) and staining results in *AtUGT* mutant. In particular, glycosylation catalyzed by a superfamily of GTs plays significant roles in modulating the solubility, stability, bioavailability and bioactivity of various small molecules (Bowles *et al.* 2006). This plasticity depends on the integration of growth, development, and metabolism, and the evolution of diverse mechanisms to regulate cellular homeostasis. These modifications help in adaption of the plants to fit better in the environment (Mishra *et al.* 2013). In this study, for the first time, we have demonstrated that expression of *Arabidopsis* UGT genes enhanced the adaptation of the *p35S:At1g43620* complemented lines of *Arabidopsis* and since its expression is mainly cold inducible but we have found *At1g43620* was expressed in lower concentration of salt and short term exposure of heat. We speculate that *At1g43620* gene may be involved in modulation of sterol glycosides and acyl sterol glycosides in environmental stress of *Arabidopsis*. We investigated the germination of all the phenotypes under different salt stress conditions, *AtUGT* mutant line showed poor germination as compared to other genotypes. At 200 mM NaCl, there was no germination in mutant while complemented line of this mutant *p35S:At1g43620* germinated well in different concentration of NaCl as compared to WT. However, the germination percentage of mutant was significantly lower at all the concentrations of NaCl, indicating that *At1g43620* gene is involved in the modulation of ratio of sterol/sterol glucoside during seed germination (Bush and Grunwald 1997). Other phenotypic characters like root length, fresh weight, dry weight and chlorophyll content were relatively less than other phenotypes in knockout mutant, while *p35S:At1g43620* lines showed comparatively better response than WT. In the context of photosynthesis, chlorophyll is an essential factor as a driving force for plant growth and biomass production.
for the maintenance of plant growth and development. Chlorophyll fluorescence of PSII (Fv/Fm) is useful tool for the monitoring of different environmental stresses. In a previous report, the physiological changes have been measured by photoinhibition at high salinity (Lu et al. 2002). This concept was strongly supported by our results. When plants were exposed to salt-stress, their reaction centers were damaged (photochemically inactive), thus reducing electron transport capacity in PSII. Similar results were previously reported in barley (Kalaji et al. 2011). In our study, both light and dark reactions of photosynthesis were impaired much more in knockout mutants than in p35S:At1g43620 complemented lines and WT Arabidopsis, since the performance indices of photochemical Y (NPQ) reactions were much lower in stress-exposed plants than in control plants.

Presence of heat shock showed a rapid increase of SG biosynthesis in the slime mould Physarum polycephalum, the yeast P. pastoris and cultured human fibroblasts. In addition, cholesteryl β-glucoside exogenously added to human fibroblasts activated the heat shock transcription factor 1 to bind to a heat shock element in front of the heat shock protein 70 gene, inducing its transcription (Grille et al. 2010). In the present study knockout mutant was severely damage under heat stress due to maximum accumulation of ROS, since MDA level was also enhanced. Whereas in complemented line MDA level was comparatively less accumulated. As evident maximum chlorophyll loss in mutant because leaves were more bleached comparatively with WT and complemented lines. Due to this reason reaction centers were damaged and fluorescence response (Fv/Fm) also decreased. It can be understood that SG plays an important role during heat stress.

The sterol content of plant membranes has been observed to change in response to environmental conditions and it has been suggested that alterations in the sterol composition of the plasma membrane may play a role in the cold acclimation process. Thus, a key function of CA (cold acclimation) is to stabilize those membranes via multiple mechanisms. For example, alterations in membrane lipid composition are correlated with membrane cryostability (Lee et al. 2010). It has been reported that the concentration of membrane sterols increases during cold acclimation in tolerant rye cultivars (Grille et al. 2010). In the present study, transcript level of AtUGT genes, At1g43620 was elevated in WT Arabidopsis, within 24 h under cold stress and during freezing tolerance test. Expression of cold responsive gene (cor15a
and rd29a) also enhanced in complemented lines as compared to mutant lines also supported our results. These observations were supported by phenotypic characterization in NA and CA studies. Survival percentage of p35S:At1g43620 lines upto 78% in cold acclimatization condition which was more than WT and also the other phenotypes. In cold stress, the membrane sterols play very important role. In wheat, ESTs corresponding to several enzymes involved in sterol metabolism are overexpressed, suggesting major lipid modifications in membranes during cold acclimation (Houde et al. 2006). Chlorophyll fluorescence imaging offers the additional advantage of providing spatial information about freezing damage (Ehlert et al. 2008). During cold stress, the Fv/Fm ratio, as well as Y (II) was reduced indicating a structural and functional disorder of the photosynthetic apparatus and damage to the PSII. In our study, AtUGT plants showed lowering of Fv/Fm as compared to other genotypes. Reductions in the Fv/Fm ratio and Y (II) under cold stress suggested that an important portion of the PSII reaction centre was damaged in the knockout mutant. These damages were associated with structural modifications in PSII.

In conclusion, our results demonstrated that expression of At1g43620 gene was enhanced during cold stress but this gene was not induced impressively at higher concentration of salt and heat stress. To address this we determined the expression profile of native At1g43620 gene in (WT plants) and two cold responsive genes (cor15a and rd29a) in (WT, mutant and complemented lines) during a time course. Cor15a and rd29a are the representative members of COR gene “regulon” and have been shown by numerous reports to be induced in Arabidopsis during cold acclimation/cold stress (Jaglo-Ottosen et al. 1998; Kasuga et al. 1999). Moreover, global cold acclimation studies proved that At1g43620 gene was strongly involved in cold acclimation of the complemented lines. However, almost nothing is actually known about their structural modification under different abiotic stress conditions which needs further research.